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TITLE:

STATUS OF SOLID-PHASE ENZYME IMMUNOASSAYS

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SUBMITTED TO:

<u>In</u>: Proceedings of the Fourth Aspen Conference in Clinical Laboratory Medicine, to be held in Aspen, Colorado (August 23-25, 1978)

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STATUS OF SOLID-PHASE ENZYME IMMUNOASSAYS

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INTRODUCTION

Solid-phase (or heterogeneous) enzyme immunoassays (EIAs) for a variety of antibodies, antigens, and haptens are being developed at a very rapid rate. Because of their basic simplicity, sensitivity, and accuracy, at least in this writer's view, they probably will replace many of the common types of immunoassays currently in use. That is, such fun-to-do procedures as complement fixation, hemagglutination, hemagglutination inhibition, and immunoprecipitation may become classroom curiosities. Indeed, even immunofluorescent and radioimmunoassays soon should be gradually replaced by solid-phase EIAs, as is already occurring with homogeneous EIAs in the hapten area.

Why are solid-phase EIAs becoming so popular? Their attributes can be listed as follows: (1) they are primary binding assays and do not require the participation of secondary events (e.g., complement fixation, agglutination, etc.) to obtain a readable result; (2) solid-phase EIAs are technically easy to perform as they employ simple incubation and wash protocols; (3) the enzyme conjugates are stable for prolonged periods (usually indefinitely) and are relatively inexpensive; (4) the versatility of the assays ranges from quick antibody screening tests to very sensitive, quantitative assays of haptens; (5) test results can be read with simple, inexpensive colorimeters or spectrophotometers; (6) the simplicity, sensitivity, and lack of need of expensive isotope counting equipment allow these assays to be performed in small, community-type hospitals; and (7) when needed, the methodology can be easily automated.

The remainder of this chapter will describe the principles and applications of the major types of solid-phase EIAs now in use. In addition, some operational considerations and a discussion of the "art" of performing these

assays will be included. Finally, a brief description of an available automated EIA system will be given. The recent excellent review by Schuurs and Van Weemen (1) is recommended to readers who require more information concerning specific assay parameters.

DESCRIPTION AND APPLICATIONS OF SOLID-PHASE ENZYME IMMUNOASSAYS

Indirect Solid-Phase EIAs.--Indirect solid-phase EIAs are most often used to detect serum antibody to antigens derived from infectious and parasitic disease agents. The binding sequence for an indirect solid-phase EIA is illustrated in Fig. 1. Antigen is first bound to the solid phase (e.g., 96-well microplate); this is usually accomplished by simple adsorption using a special adsorption buffer (see Table I). Suspect serum is then incubated with the antigen and, if the serum contains antibody, it will bind to the adsorbed antigen as illustrated (Fig. 1). The incubation time varies with the assay but ranges from just a few minutes to several hours. After incubation, nonbound serum is removed by several washes, and then enzyme-labeled antispecies antibody is added where it binds (in the case of a positive serum sample) to the antibody already attached to the antigen. The length of conjugate incubation is also quite variable from assay to assay. Following another wash sequence, substrate is provided for the bound enzyme which, in turn, is catalyzed to form a colored reaction product. The test result can be read visually or can be digitized by colorimetric or spectrophotometric means. Because the conjugates are directed toward bound antibody, one conjugate can be used for a variety of etiologic agents which infect a given species. Table II presents an abbreviated list of some of the diseases for which indirect EIAs have been developed.

Double-Antibody Solid-Phase Enzyme Immunoassay.--Double-antibody solid-phase EIAs can be used to detect large molecular weight antigens such as bacterial toxins, soluble protein antigens from infectious agents, and viruses. In theory, at least two epitopes must be present per molecule of antigen. In practice, however, the sensitivity of the assay should increase in relation to the number and distribution of epitopes available. The binding sequence of a double-antibody solid-phase EIA is illustrated in Fig. 2. First, antibody (as specific and pure as possible) to the antigen in question is bound to the solid phase, again usually by adsorption techniques but occasionally by covalent linkage (15,16). Suspect material is then incubated with the adsorbed antibody (15 minutes to several hours) and, if antigen is present, it will bind to its antibody. Following a wash sequence, antibody conjugated to an enzyme is added to the reaction vessel. If antigen was bound during the prior incubation, the conjugated antibody should bind to the remaining epitopes. After another wash sequence, substrate is provided, and the resulting reaction product is quantitated colorimetrically or spectrophotometrically. Table III lists some of the published applications of double-antibody solid-phase EIAs.

Competitive Binding Solid-Phase Enzyme Immunoassay.--Competitive binding type solid-phase EIAs can be used to quantitate both macromolecular and haptenic substances. For haptens, one must devise a suitable chemical procedure for linking the molecule to the enzyme such that hapten specificity for its antibody is not altered. One must also consider that alteration of the affinity constant for antibody of labeled vs nonlabeled molecules is possible.

The binding sequence for competitive binding EIAs is illustrated in Fig. 3. First, antibody is bound to the surface of the solid phase (e.g., a microplate). Enzyme-labeled antigen is added to one set (one or more) of wells, while enzyme-labeled antigen plus unlabeled "unknown" antigen is added to a second set of

wells. After a suitable incubation period, the reaction vessels are washed, and substrate is added to the wells. Again after a suitable incubation period, the reaction is stopped, and the absorbance of the reaction product present in each well is determined. The amount of unknown antigen present is determined by subtracting the absorbance of the unknown from the absorbance of labeled antigen only and plotting the result against a standard curve. Table IV lists several applications of competitive binding EIAs.

OPERATIONAL CONSIDERATIONS

Choice of the Enzyme Amplifier.--A list of enzymes used as amplifiers for heterogeneous EIAs is presented in Table V. The most commonly used enzymes have been alkaline phosphatase, β-galactosidase, and horseradish peroxidase (HRP). A single enzyme may not be suitable for every EIA performed at any single laboratory. Criteria used in choosing an enzyme amplifier for a particular assay include (1) turnover number (how fast does the enzyme convert substrate); (2) sensitivity and safety of available substrate systems (certain chromogens used with HRP are carcinogenic such as diaminobenzidene); (3) ability to couple the enzyme to the desired molecule without greatly altering enzyme activity; (4) stability of the conjugate produced; (5) availability and cost of the enzyme; (6) possible interference of test fluids with the enzyme system (for example, free hemoglobin may nonspecifically bind to the solid phase where it can catalyze substrates used in HRP EIAs); and (7) molecular weight of the conjugates obtained. This becomes especially important if the conjugate must penetrate cell membranes, as in indirect cell-bound virus EIAs [e.g., hog cholera (11)].

Choice of Solid Phase.--A variety of materials and configurations have been used as solid phase, some of which are listed in Table VI. Probably the most commonly used reaction vessel for manual testing is the polystyrene or polyvinyl

microtitration tray as it is inexpensive, convenient to use, readily available and, most important, appears to work well for most assays. In the Technicon automated system to be described below, small polystyrene tubes are employed.

Binding of Antigen and Antibodies to the Solid Phase.--In most assays, binding is accomplished through various adsorptive processes, although covalent linkage has been employed (15,16). The three methods we have used in our work are (1) direct drying of the antigen or antibody to the solid phase (4); (2) wet application of antigen or antibody using a special coating buffer (2); and (3) pretreatment of solid phase with bovine serum albumin (18). In the first method, one simply dilutes the antigen (or antibody) appropriately and then applies an aliquot (usually 50 to 100 µl) to each tube or microplate well, where it is allowed to air-dry. For the second method, the antigen (or antibody) is diluted in the coating buffer (see Table I for composition of the buffer) and aliquoted into the solid phase, which is then sealed to prevent evaporation. Adsorption is allowed to continue for several hours or longer, and the solution can be kept in the reaction vessel until use. In the final method, BSA (200 mg/l H_2O) is aliquoted in the amount of 50 µl per reaction well or tube, allowed to air-dry, and is then fixed with a 0.25% glutaraldehyde solution for 30 minutes. After thorough washing, 50- to 100-µl aliquots of diluted antigen (or antibody) are added and allowed to air-dry. The procedure of choice will vary with the assay; it is best, therefore, to try each of these methods for each new assay to determine which one is most efficient. The stability of prepared antigen carriers varies somewhat but usually is on the order of months.

Nonspecific Binding: How to Deal with It.--Nonspecific binding (NSB) of immunoglobulin, conjugate, or interfering proteins (e.g., hemoglobin in HRP-based assays) to the solid phase can reduce both EIA sensitivity and specificity, especially those of the indirect type. Binding of these materials to the solid

phase is facilitated by aggregation or other denaturation of protein, the presence of large immune complexes and, to some degree, can be related to the relative amounts of protein present in the sample. During incubation periods, all proteins will bind somewhat to available sites remaining on the solid phase. With long incubation periods, all available sites eventually will be occupied by one molecular type or another. Methods to reduce nonspecific binding are designed both to occupy remaining binding sites (after adsorption of either antigen or antibody) and to prevent the firm adsorption of undesired molecules. The first step of an assay often will include a preliminary incubation step with an indifferent protein such as BSA, or the diluted test fluid may contain BSA in fairly high concentration in relation to potentially interfering proteins. These procedures are designed to bind preferentially innocuous molecules to free sites, thus significantly reducing the binding of molecules which can add "noise" to the system. In addition, a nonionic detergent (e.g., Tween 20 or Tween 80) is usually added to incubation fluids as a deterrent to NSB. In our work, we have found that a mildly alkaline, high ionic strength buffer containing Tween 80, when used as a diluting fluid for both serum and conjugate, works very well to prevent significant NSB. The composition of this buffer is 0.5 M NaCl, 0.5% Tween 80, and 0.01 M PO_4, final pH 8.0.

Miscellaneous Considerations.--As in all immunoassays, EIAs are dependent on the quality of reagents for their sensitivity and specificity. Where possible, purification procedures should be applied to antigens, antibodies, and conjugates so that precise and accurate results occur with their use. When quantitative assays are performed, it is important also that the reaction product generation follows zero order kinetics. As EIAs often employ microquantities (50 to 100 µl) of reagent, deviation from zero order kinetics can occur quite rapidly when significant amounts of enzyme have been bound (due to substrate

depletion and/or reaction product inhibition). Finally, methods must be devised to standardize EIA reagents (especially conjugates) such that various laboratories using the same or similar reagents can obtain the same results. More about this important area can be found elsewhere in this volume (30).

AUTOMATION OF SOLID-PHASE ENZYME IMMUNOASSAYS

A collaborative effort among the Los Alamos Scientific Laboratory, Technicon Instruments Corporation, and the United States Department of Agriculture has resulted in a totally automated processor for indirect solid-phase EIAs capable of processing up to 300 samples per hour (31). With minor modifications, the system can be adapted to sandwich and competitive binding types of EIAs. The configuration of the system is illustrated both photographically (Fig. 4) and schematically (Fig. 5).

Principle of Operation.--Like all Technicon analytical instruments, continuous flow principles are relied on heavily during sample processing. Reference to both Figs. 4 and 5 will aid the reader in the following discussion. Processing rates, incubation times for serum, conjugate and substrate, number of washes, and sample-to-wash ratio are first programmed into the controller at 4 (Fig. 5). There are two channels, each of which is processed in the same manner. Cups of undiluted samples are loaded into the primary sampler at 13, and the sample is picked up and moved through a tube to the proportioning pump at 11, where it is diluted and mixed. The sample then proceeds to the incubation sampler at 12 and is deposited into an antigen-containing tube. Serum incubation, serum wash, conjugate application and incubation, conjugate wash, substrate application and incubation, and reaction product pickup are all performed in the incubation sampler, which contains a temperature-controlled water bath. After

reaction product pickup, the sample is transported through the proportioning pump at 10, where it is diluted before it proceeds through a colorimeter at 5 and 6 and thence to waste. The signal obtained from the colorimeter is transmitted to the recorder 3, where a graphical readout of each sample occurs. Positive samples are those whose signal in chart divisions is greater than a threshold value determined by running standard negative-control samples. Figure 6 diagrammatically illustrates the sample pathway through the EIA system.

SUMMARY

Solid-phase enzyme immunoassays are becoming increasingly popular due to their sensitivity, simplicity, and versatility. The three most common types of these assays (indirect, double-antibody, and competitive binding) have been described and examples given of their use. Short discussions of various aspects of the "art" of performing solid-phase EIAs have been included. Finally, a brief overview of the Technicon automated EIA processor has been presented.

ACKNOWLEDGMENT

This work was supported by the United States Department of Agriculture (Animal and Plant Health Inspection Services) and the United States Department of Energy under an interagency agreement.

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TABLE I. Preparation of Coating Buffer (2) for Antigen Adsorption

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.20 g
Distilled H_2O	1000 ml

TABLE II. Selected Applications of Indirect Solid-Phase EIAs for the Detection of Antibody

Antibodies against	Reference
Trichinella spiralis	3,4
Echinococcus granulosus	5
Plasmodium species	5
Schistosoma mansoni	6
Trypanosoma species	6
Brucella abortus	4,7
Escherichia coli	8
Vibrio cholerae	9
Typhus rickettsiae	10
Hog cholera virus	4,11
Herpes simplex virus	12
Rubella virus	13
DNA (double-stranded)	14

TABLE III. Selected Applications of Double Antibody Solid-Phase EIAs

Antigen detected	Reference
IgG	15
Factor VIII	2
Alpha-fetoprotein (rat and human)	17
Staphylococcus enterotoxin A	18
Human chorionic gonadotropin	19
Hepatitis B surface antigen	20
Herpes simplex virus	21

TABLE IV. Selected Applications of Competitive Binding EIAs

Antigen detected	Reference
IgG	22
Alpha-fetoprotein	23
Carcinoembryonic antigen	24
Human chorionic gonadotropin	19
Insulin	25
Thyroid stimulating hormone	26
Cortisol	27
Gentamicin	28
Aflatoxin B_1	29

TABLE V. Enzyme Labels Used for Solid-Phase EIAs

Acetylcholinesterase

Alkaline phosphatase

β-D-Galactosidase

Carbonic anhydrase

Glucoamylase

Glucose oxidase

Horseradish peroxidase

TABLE VI. Solid Phases Used in EIAs

Cellulose acetate discs

Polystyrene and polyvinyl tubes, discs, beads, and microplates

Glass rods and beads

Tissue culture cells

Sepharose beads

Fig. 1. Binding sequence for indirect solid-phase EIAs.

INDIRECT SOLID PHASE EIA

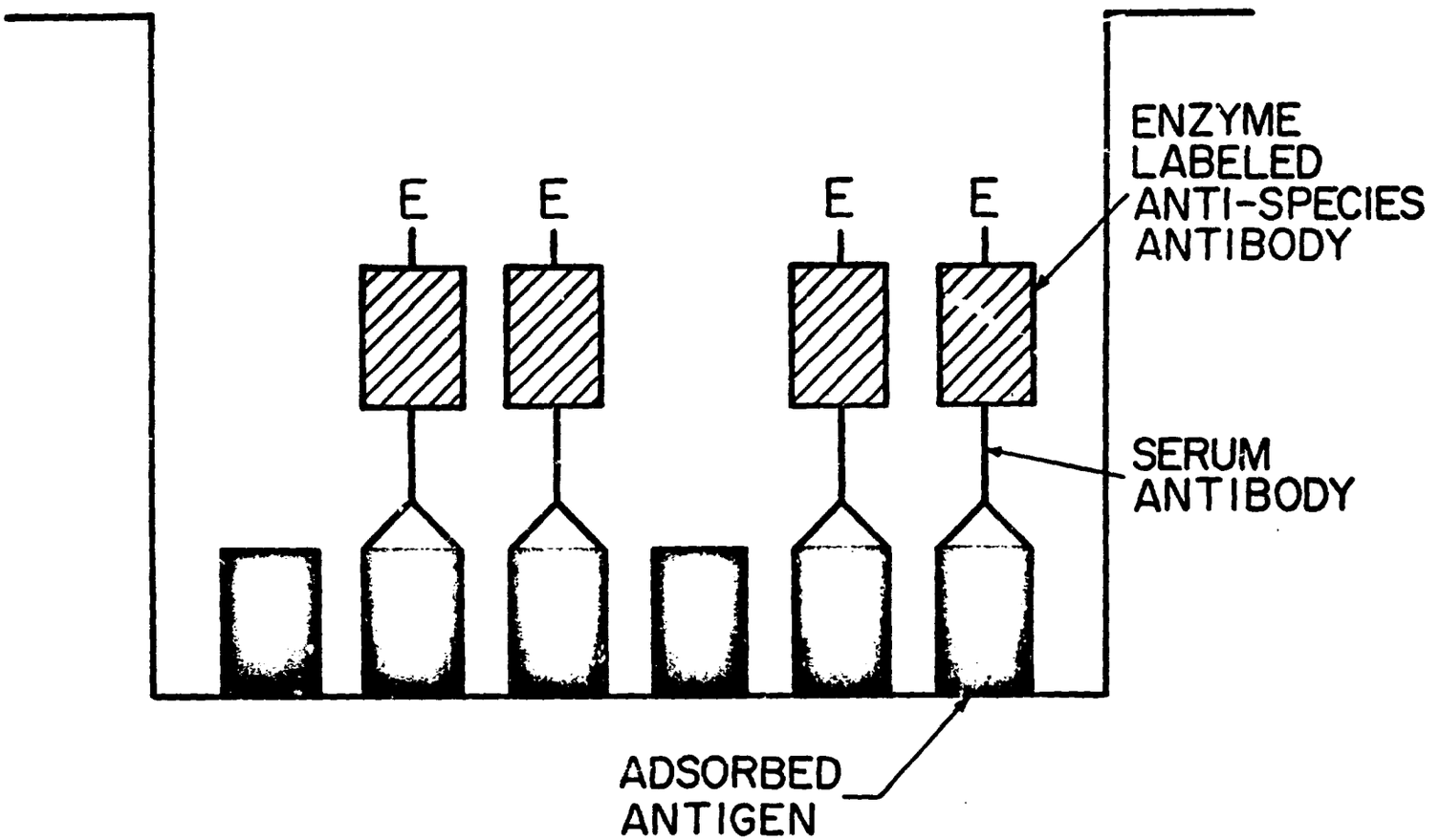


Fig. 2. Binding sequence for double-antibody solid-phase EIAs.

DOUBLE ANTIBODY SOLID PHASE EIA

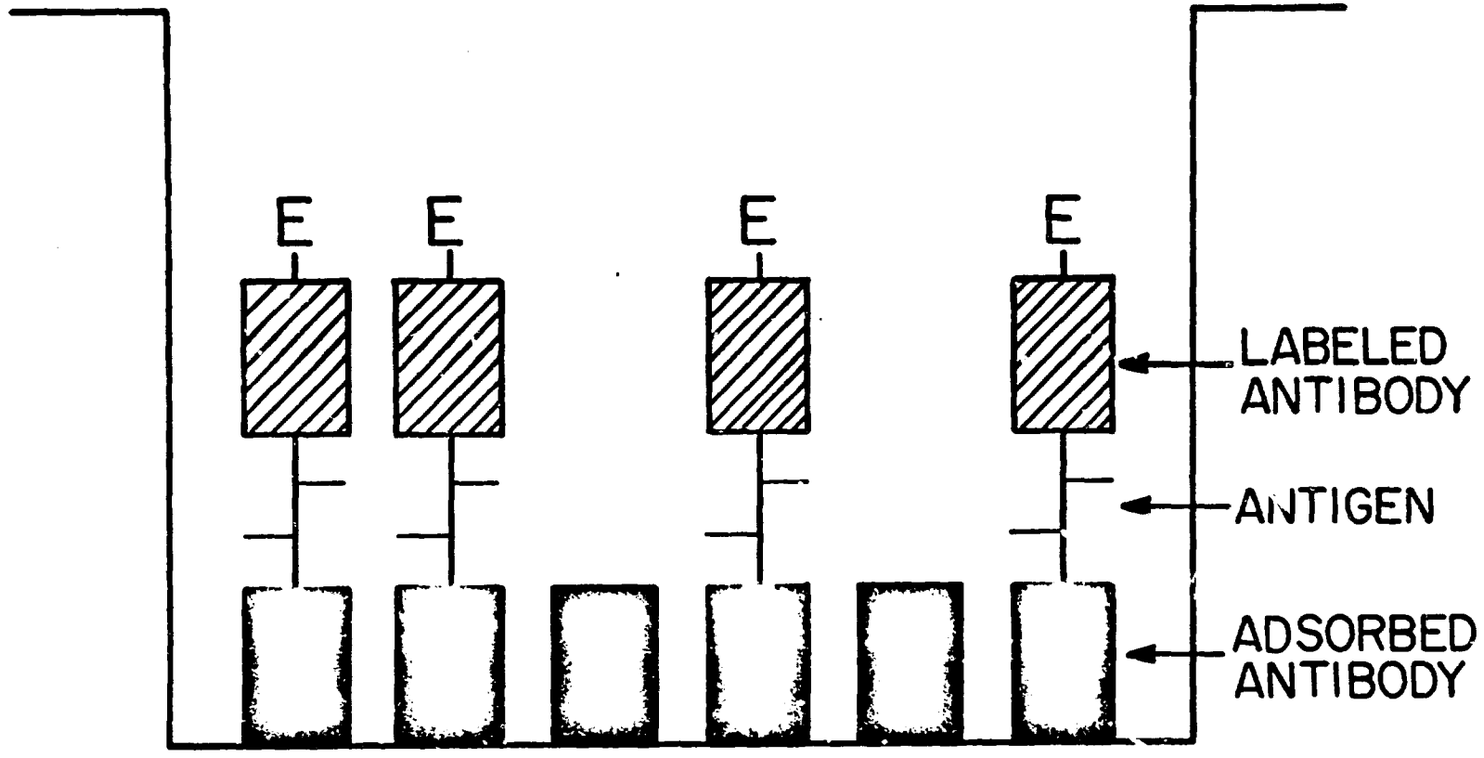


Fig. 3. Binding sequence for competitive binding EIAs.

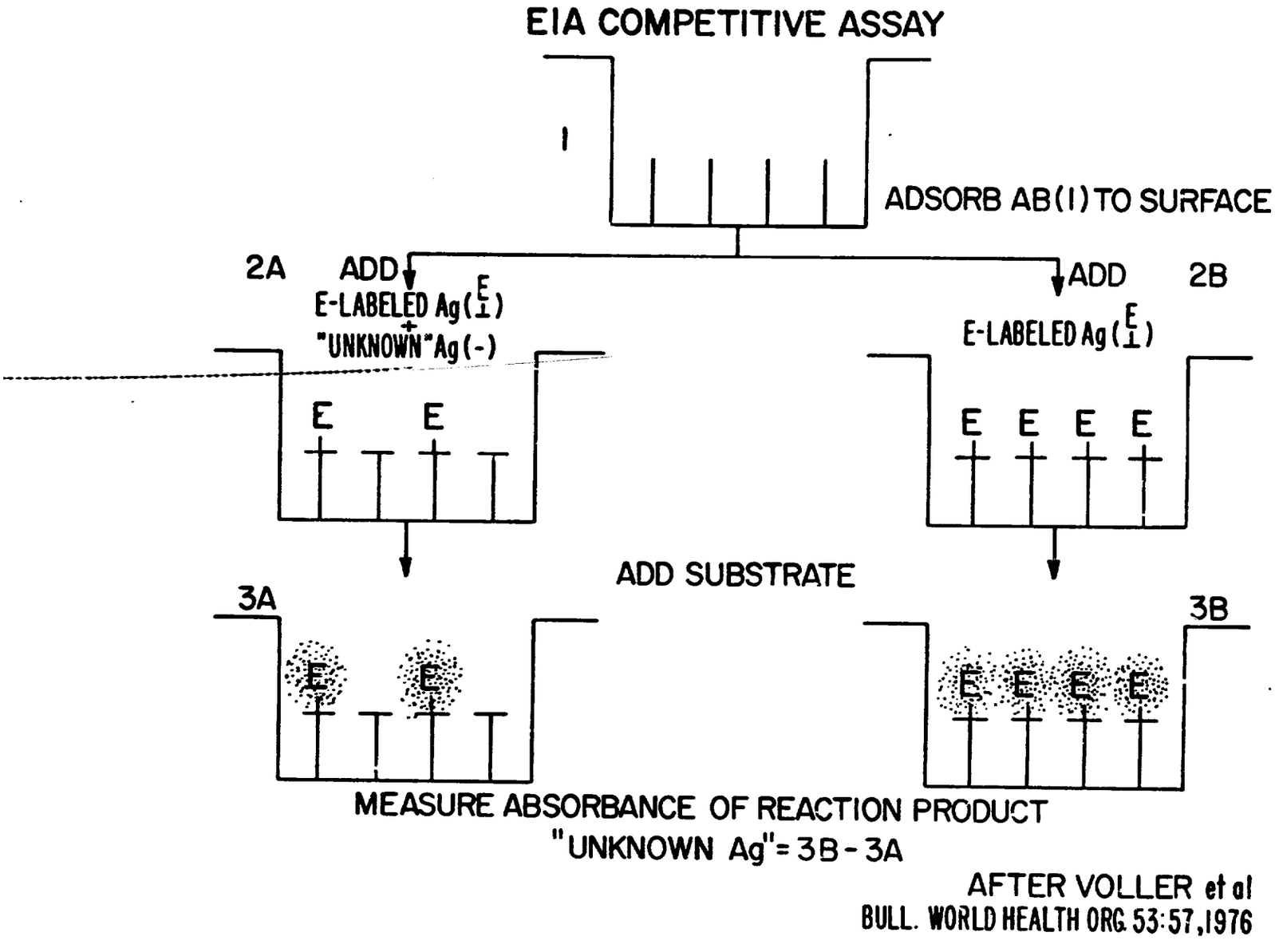

EIA COMPETITIVE ASSAY
1
ADSORB AB(1) TO SURFACE
2A
ADD
E-LABELED Ag(E1)
+
"UNKNOWN" Ag(-)
ADD
2B
E-LABELED Ag(E1)
E
E
E
E
E
E
ADD SUBSTRATE
3A
3B
MEASURE ABSORBANCE OF REACTION PRODUCT
"UNKNOWN Ag" = 3B - 3A
AFTER VOLLER et al
BULL. WORLD HEALTH ORG. 53:57, 1976

Fig. 4. Photograph of the Technicon automated EIA processor (photograph courtesy of Technicon Instruments Corporation, Tarrytown, N. Y.).

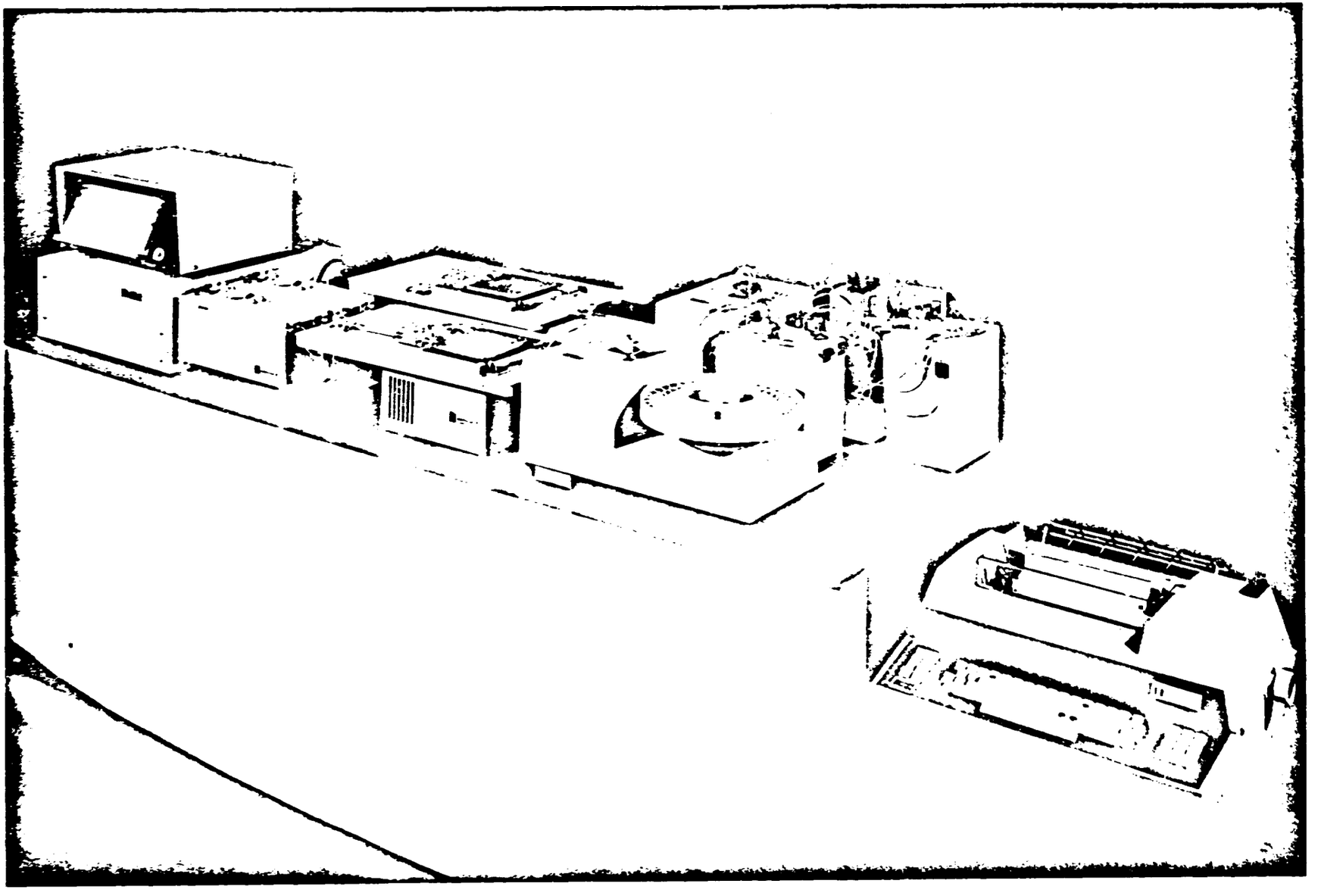
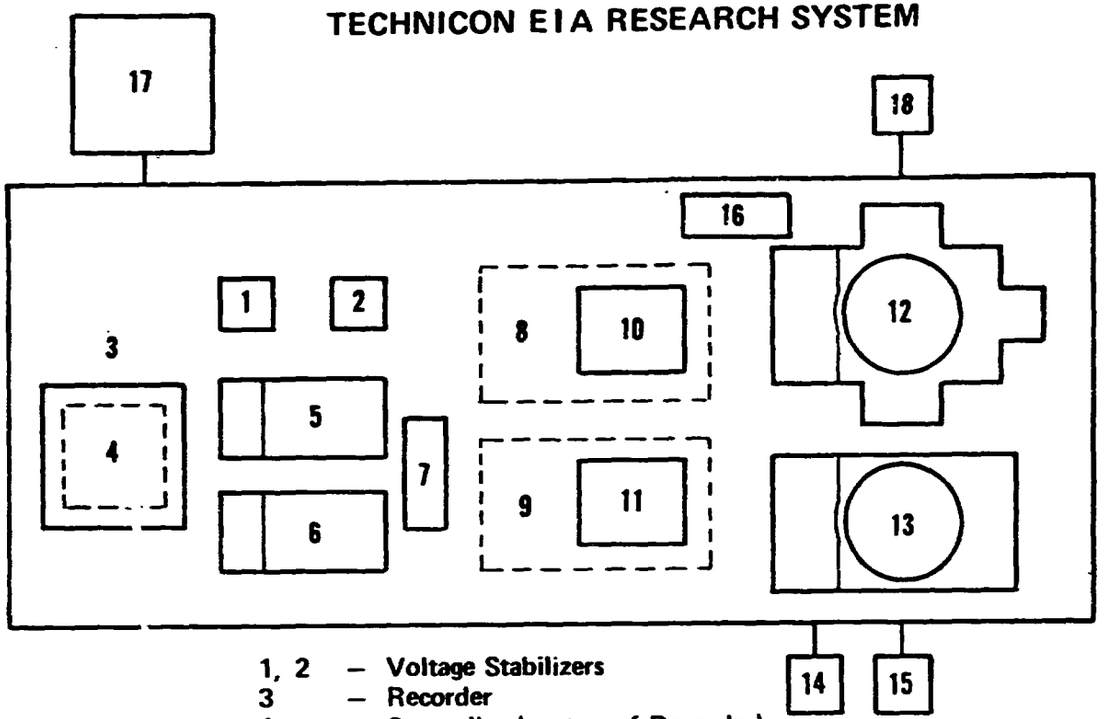


Fig. 5. Schematic representation of the Technicon automated EIA processor (courtesy of Technicon Instruments Corporation, Tarrytown, N. Y.).

TECHNICON EIA RESEARCH SYSTEM



1, 2 – Voltage Stabilizers
3 – Recorder
4 – Controller (on top of Recorder)
5, 6 – Colorimeters
7 – Waste Tray
8 – Platter Manifold (Wash) (On top of Pump)
9 – Platter Manifold (Reagent) (On top of Pump)
10,11 – Proportioning Pumps
12 – Incubation Sampler
13 – Primary Sampler
14 – Haake H_2O Bath (under bench)
15 – Vacuum Pump (under bench)
16 – Hydraulics Module
17 – Teleprinter (optional) (floor model)
18 – Electrical Outlet Strip (under bench, preferably)

Fig. 6. Diagrammatic illustration of the sample pathway through the Technicon EIA processor (courtesy of Technicon Instruments Corporation, Tarrytown, N. Y.).

SAMPLE PATHWAY THROUGH E I A SYSTEM